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Determination of biochemical baseline of resistance against bacterial leaf spot of chilli after application of plant defense activators

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Present study was designed to determine the progressive alterations in the leaves of chilli after the application of plant activators to pave the way towards fruitful management of bacterial leaf spots of chilli. Different plant defense activators such as KH_2PO_4, K_2HPO_4 and salicylic acid were used and alterations in the biochemicals of chilli were quantified by following standard protocols. In the first experiment, KH_2PO_4, K_2HPO_4 and salicylic acid were evaluated at a different concentration under field conditions. Application of salicylic acid expressed less disease incidence (21.111), followed by K_2HPO_4 (25.167) and KH_2PO_4 (28.889) as compared to the control. Alterations in biomolecules (SOD, POD, CAT, H_2O_2, TSS and TSP) were quantified with the application of salicylic acid (0.9285, 0.9297, 0.9347, 1.2278, 0.6663, 0.6804 and 0.6723) followed by K_2HPO_4 (0.6502, 0.6605, 0.6544, 0.7689, 0.5122, 0.5322 and 0.5222) and KH_2PO_4 (0.4729, 0.4713, 0.4778, 0.4522, 0.3544, 0.3744 and 0.3644) µg/g. Randomized Complete Block Design (RCBD) was used for the field experiments.
Keywords: Salicylic acid, KH_2PO_4, K_2HPO_4, Peroxidase, Total soluble phenols, Total soluble sugars.

INTRODUCTION

Chilli (*Capsicum annum L.)* is an imperative crop which is grown in all provinces of Pakistan (Ali *et al.*,2020). Numerous strategies like chemical, biological, and cultural control and resistant varieties have been employed to control the bacterial leaf spot of chilli. The most economical, eco-friendly, and effective management strategy is the use of resistant sources but *Xanthomonas campestris* pv. *vesicatoria* is responsible for withdrawing various varieties in chilli cultivation worldwide under conducive environmental conditions. Plant disease management commonly relies on synthetic chemicals that are potentially harmful to the environment and human beings (Kalia *et al.*, 2020). Although use of bio-control agents is promising but development of bio-formulated products needs extensive field evaluation and various biocontrol agents are not likely to thrive in new habitats (Rajput *et al.*, 2021). Therefore, the application of plant activators can serve as a greener option to overcome the bacterial leaf spot of chilli. Plant defense activators, activate the host plant defense genes, which trigger the induced resistance (IR) by initiating signals through the signal transduction pathway, which provides long-lasting protection against a destructive and extensive range of pathogens (Ahmad *et al.*, 2010). Salicylic acid plays a key role in regulating plant growth and development. It has been investigated that salicylic acid mediates the oxidative burst. Plant activators specifically bind to CAT and minimize its activity. Salicylic acid can increase H_2O_2 levels in plant cells and potentially induce antioxidant enzyme expression, enhancing plant tolerance to biotic and abiotic stress (Szepesi *et al.*, 2008). Biomolecules play a vital role in plant-disease interactions.

Plants become prone to infection due to alterations in enzymatic and non-enzymatic metabolites that aid the plants in restraining the pathogens. Quantification of these alterations will help researchers to develop concrete solution for disease management (Hameed *et al.*, 2021). In the present study, the resistance baseline against bacterial leaf spots of chilli was determined by quantifying alterations in

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biochemical compounds after the application of plant defense activators. Outcomes of the present study can be used as biochemical markers for identification of resistant sources against *Xanthomonas campestris* pv. *vesicatoria* and are helpful to develop an eco-friendly management strategy towards bacterial leaf spots of chilli.

MATERIALS AND METHODS

***Evaluation of plant activators against Xanthomonas campestris pv. vesicatoria under field conditions*:** Chilli seedlings were transplanted in the field and arranged under RCBD with four replications under field conditions (5×3 m^2). Recommended horticultural practices were maintained, including number of irrigations to keep the plants healthy. Aqueous suspension of the bacteria was prepared from 48 hours old actively growing culture and bacterial concentration was adjusted @ 1×10^8 CFU/mL with the help of spectrophotometer (Hitachi U-2001, model 121003). Bacterial suspension was inoculated early in the morning (when maximum number of stomata were opened) in the leaves of chilli plants with the help of syringe method (Atiq *et al*., 2022). Three plant defense activators like K_2HPO_4, KH_2PO_4 and salicylic acid @ 0.5, 0.75 and 1% along with control (distilled water) were evaluated by using spray method (Atiq *et al*., 2022) and data regarding incidence of leaf spot of chilli was noted after 7,14 and 21 days of application of plant defense activators.

***Sample preparation for biochemical analysis*:** Chilli leaves were collected from treated and untreated plants and were cut into small pieces. 0.5 g of leaf sample was taken and ground in pestle and mortar along with KH_2PO_4 buffer. These samples were centrifuged (Horizon 6 Flex) at 12000 rpm for 5 minutes and the supernatant was collected to analyze biochemical compounds (TSP, SOD, POD, CAT, TSS, TPC).

***Determination of SOD (superoxide dismutase) from un-treated and treated chilli leaves*:** Quantification of SOD from leaves of chilli was done by preparing the reaction mixture by mixing 100 µL enzyme extract, methionine (200 µL), NBT (100 µL), triton X (200µL), potassium phosphate buffer (500 µL, pH 5) and distilled water (800 µL). The mixture was placed for fifteen minutes under UV (ultraviolet light) for 15 minutes. After this, 100 µL riboflavin (Vitamin B2) was added and absorbance was estimated through a spectrophotometer (Hitachi U-2001: 121-003) at 560 nm (Giannopolitis and Ries, 1977).

***Determination of POD from un-treated and treated chilli leaves*:** To estimate Peroxidase (POD) from the leaves of chilli, a reaction mixture was made by mixing enzyme extract (100 µL), 18 mM guaiacol (100 µL), KH_2PO_4 buffer (800 µL, pH 5) and 42 mM hydrogen peroxide (100 µL). Absorbance was estimated at 470 nm through a spectrophotometer (Liu *et al*., 2007).

***Determination of catalase (CAT) from un-treated and treated chilli leaves*:** To determine catalase (CAT) from the leaves of chilli, a reaction mixture was prepared by mixing enzyme extract (100 µL) and 100 µL of 5.95mM hydrogen peroxide. Absorbance was recorded at 240 nm using a spectrophotometer (Liu *et al*., 2007).

***Estimation of H_2O_2 Concentration from un-treated and treated chilli leaves*:** To estimate H_2O_2 concentration, fresh leaves were collected from untreated and treated chilli leaves. A sample of the fresh leaf (50 mg) was taken and ground within a ($C_2HCl_3O_2$) buffer and centrifuged at 12000 rpm for 15 minutes at 4 °C. After this, 1.3mL of potassium phosphate buffer (pH 7) and 1mL of potassium iodide was mixed with 0.3mL supernatant and incubated (incubator, RTI-250) for 5 minutes. The filtrate was firstly treated with KH_2PO_4 (pH 7) buffer and then with KI. The resultant mixture was placed in a digital incubator (RTI-250) for 5 minutes and their absorbance was taken at 390 nm through an absorbance reader (BioTek: 800TS) and the amount of hydrogen peroxide (H_2O_2) was taken as $\mu mol \cdot g^{-1}$ FW (Velikova *et al*., 2000).

***Estimation of TPC (total phenolic content) from un-treated and treated chilli leaves*:** To determine TPC, an enzyme extract (100 µL) was prepared. The reaction mixture consisted of 200 µL F-C reagent (10%), 700 mM Na_2Co_3 800 µL and was left for 1 hour. Absorbance was estimated through a spectrophotometer at 765nm (Barba *et al*., 2013).

RESULTS

***Effect of plant activators against Xanthomonas campestris pv vesicatoria under field condition*:** Among all the treatments, salicylic acid (21.111) expressed less disease, followed by K_2HPO_4 (25.167) and KH_2PO_4 (28.889) % as compared to the control (Table 1). Treatments and concentration interaction (T×C) indicated that salicylic acid expressed minimum disease incidence (22.167, 21.333 and 19.833), followed by K_2HPO_4 (26.333, 25.167 and 24.000) and KH_2PO_4 (30.167, 29.000 and 27.500) % @ 0.5, 0.75 and 1% as compared to control (Table 2).

Table 1. Evaluation of plant defense activators against leaf spot of chilli under field conditions.

Treatments	Disease incidence (%)
KH_2PO_4	28.88 b
K_2HPO_4	25.16 c
Salicylic acid	21.11 d
Control (distilled water)	70.00 a
LSD	3.35

*Mean value in the column sharing a similar letter does not differ significantly as determined by the LSD test ($P < 0.05$).

Effect of plant defense activators on the biochemical compounds in chilli leaves

***Effect of plant defense activators on SOD under field condition*:** Among all the treatments, salicylic acid (0.9285) expressed more SOD value followed by K_2HPO_4 (0.6502) and KH_2PO_4 (0.4729) µg/g as compared to the control (Table 3). Treatments and concentration interaction (T×C) indicated that salicylic acid expressed maximum SOD value (0.7417, 0.903 and 1.1137) followed by K_2HPO_4 (0.5131, 0.6167 and 0.8207) and KH_2PO_4 (0.3342, 0.4524 and 0.6322) µg/g at 0.5, 0.75 and 1% concentrations as compared to the control (Table 4).

Table 2. Evaluation of plant defense activators and their concentrations towards leaf spot of chilli under field conditions.

Treatments	Disease incidence (%) Concentrations (%)		
	0.5%	0.75%	1%
KH_2PO_4	30.17b	29.00bc	27.50bcd
K_2HPO_4	26.33b-e	25.17b-f	24.00c-f
Salicylic acid	22.17def	21.33ef	19.83f
Control (dist. water)	70.00a	70.00a	70.00a
LSD		3.802	

*Mean value in the column sharing a similar letter does not differ significantly as determined by the LSD test (P < 0.05).

Table 3. Evaluation of plant defense activator against leaf spot of chilli under field conditions.

Treatments	Superoxide dismutase (SOD) µg/g
KH_2PO_4	0.4729 c
K_2HPO_4	0.6502 b
Salicylic Acid	0.9285 a
Control (dist. water)	0.2500 d
LSD	0.0973

*Mean value in the column sharing a similar letter does not differ significantly as determined by the LSD test (P < 0.05).

***Effect of plant defence activators on POD under field conditions*:** Among all the treatments, salicylic acid (0.9297) expressed more POD value followed by K_2HPO_4 (0.6502) and KH_2PO_4 (0.4713) µg/g as compared to the control (Table 5). Treatments and concentration interaction (TxC) indicated that salicylic acid expressed maximum POD value (0.7417, 0.903 and 1.1170) followed by K_2HPO_4 (0.5131, 0.6167 and 0.8207) and KH_2PO_4 (0.3325, 0.4524 and 0.6289) µg/g at 0.5, 0.75 and 1% concentrations as compared to the control (Table 6).

Table 5. Evaluation of Plant defense activator against leaf spot of chilli under field conditions to check the impact of peroxidase (POD).

Treatments	Peroxidase (POD) µg/g
KH_2PO_4	0.4713 c
K_2HPO_4	0.6502 b
Salicylic Acid	0.9297 a
Control	0.2300 a
LSD	0.0981

*Mean value in the column sharing a similar letter does not differ significantly as determined by LSD test (P < 0.05).

Table 6. Evaluation of Plant defense activator against leaf spot of chilli under field conditions and to check the impact of the interaction between treatments and concentrations (T×C) on peroxidase (POD).

Treatments	Peroxidase (POD) µg/g Concentrations (%)		
	0.5%	0.75%	1%
KH_2PO_4	0.332 gh	0.452 fg	0.629 de
K_2HPO_4	0.513 ef	0.617 def	0.821 bc
Salicylic acid	0.742 cd	0.930 b	1.117 a
Control (dist. water)	0.230 h	0.230 a	0.230 a
LSD		0.1699	

*Mean value in the column sharing similar letter does not differ significantly as determined by LSD test (P < 0.05)

***Effect of plant activators on catalase (CAT) under field conditions*:** Among all the treatments, salicylic acid (0.9347) expressed more CAT value followed by K_2HPO_4 (0.6544) and KH_2PO_4 (0.4778) µg/g as compared to the control (Table 7). Treatments and concentration interaction (TxC) indicated that salicylic acid expressed maximum CAT value (0.7467, 0.9333 and 1.1240) followed by K_2HPO_4 (0.5167, 0.6200 and 0.8267) and KH_2PO_4 (0.3400, 0.4600 and 0.6333) µg/g at 0.5, 0.75 and 1% concentrations as compared to the control (Table 8).

Table 7. Evaluation of Plant defense activator against leaf spot of chilli under field condition and to check the impact of treatment on CAT.

Treatments	Catalase (CAT) µg/g
KH_2PO_4	0.478 c
K_2HPO_4	0.654 b
Salicylic Acid	0.935 a
Control (distilled water)	0.210 d
LSD	0.100

*Mean value in the column sharing similar letters does not differ significantly as determined by the LSD test (P < 0.05).

Table 8. Impact of interaction between treatments and concentrations (T×C) on CAT.

Treatments	Catalase (CAT) µg/g Concentrations (%)		
	0.5%	0.75%	1%
KH_2PO_4	0.340 gh	0.460 fg	0.633 de
K_2HPO_4	0.517 ef	0.620 de	0.827 bc
Salicylic acid	0.747 cd	0.933 b	1.124 a
Control (dist. water)	0.210 h	0.210 h	0.210 h
LSD		0.1732	

*Mean value in the column sharing similar letters does not differ significantly as determined by the LSD test (P < 0.05).

***d) Effect of plant activators on H_2O_2 under field conditions*:** Among all the treatments, salicylic acid (1.2278) expressed more H_2O_2 value followed by K_2HPO_4 (0.7689) and KH_2PO_4 (0.4522) µg/g as compared to the control (Table 9). Treatments and concentration interaction (TxC) indicated that salicylic acid expressed more H_2O_2 value (1.1767, 1.2300 and 1.1240) followed by K_2HPO_4 (0.7000, 0.7633 and 0.8433) and KH_2PO_4 (0.3800, 0.4500 and 0.5200) µg/g at 0.5, 0.75 and 1% concentrations as compared to the control (Table 10).

Table 9. Evaluation of Plant defense activator against leaf spot of chilli under field condition and to check the impact of treatment on H_2O_2

Treatments	Hydrogen peroxide (H_2O_2) µg/g
KH_2PO_4	0.452 c
K_2HPO_4	0.769 b
Salicylic acid	1.228 a
Control (distilled water)	0.230 d
LSD	0.098

*Mean value in the column sharing similar letters does not differ significantly as determined by the LSD test (P < 0.05).

Table 10. Impact of interaction between treatments and concentrations (T×C) on H_2O_2.

Treatments	Catalase (CAT) µg/g Concentrations (%)		
	0.5%	0.75%	1%
KH_2PO_4	0.387 de	0.450 d	0.520 cd
K_2HPO_4	0.700 bc	0.763 b	0.843 b
Salicylic acid	1.177 a	1.230 a	1.277 a
Control (dist. water)	0.230 e	0.230 e	0.230 e
LSD		0.1968	

*Mean value in the column sharing similar letters does not differ significantly as determined by the LSD test (P < 0.05).

***Effect of plant activators on total soluble sugars (TSS) under field conditions*:** Among all the treatments, salicylic acid (0.6663) expressed less soluble sugars value, followed by K_2HPO_4 (0.5122) and KH_2PO_4 (0.3544) µg/g as compared to the control (Table 11). Treatments and concentration interaction (T×C) indicated that salicylic acid expressed less TSS value (0.6207, 0.6660 and 0.7123) followed by K_2HPO_4 (0.4633, 0.5100 and 0.5633) and KH_2PO_4 (0.3067, 0.3533 and 0.4033) µg/g at 0.5, 0.75 and 1% concentrations as compared to the control (Table 12).

Table 11. Evaluation of Plant defense activator against leaf spot of chilli under field condition and to check the impact of treatment on total soluble sugars (TSS).

Treatments	Total soluble sugars (µg/g)
KH_2PO_4	0.354 c
K_2HPO_4	0.512 b
Salicylic Acid	0.666 a
Control (distilled water)	0.170 d
LSD	0.082

*Mean value in the column sharing similar letters does not differ significantly as determined by the LSD test (P < 0.05).

Table 12. Impact of interaction between treatments and concentrations (T×C) on total soluble sugars (TSS).

Treatments	Total soluble sugars (µg/g) Concentrations (%)		
	0.5%	0.75%	1%
KH_2PO_4	0.307 gh	0.353 fg	0.403 efg
K_2HPO_4	0.463 def	0.510 bcd	0.563 cde
Salicylic acid	0.621 abc	0.666 ab	0.712 a
Control (dist. water)	0.170 h	0.170 h	0.170 h
LSD		0.1410	

*Mean value in the column sharing similar letters does not differ significantly as determined by the LSD test (P < 0.05).

***Effect of plant defense activators on total soluble phenols (TSP) under field condition*:** Among all the treatments, salicylic acid (6.804) expressed more TSP value followed by K_2HPO_4 (0.5322) and KH_2PO_4 (0.3744) µg/g as compared to the control (Table 13). Treatments and concentration interaction (TxC) indicated that salicylic acid expressed more TSP values (0.6347, 0.6833 and 0.7233) followed by K_2HPO_4 (0.4833, 0.5300 and 0.5833) and KH_2PO_4 (0.3267, 0.3733 and 0.4233) µg/g at 0.5%, 0.75% and 1% concentrations as compared to the control (Table 14).

Table 13. Evaluation of plant defense activator against leaf spot of chilli under field conditions and to check the impact of treatment on total soluble phenols (TSP).

Treatments	Total soluble phenols (TSP) µg/g
KH_2PO_4	0.374 c
K_2HPO_4	0.532 b
Salicylic acid	0.680 a
Control (DW)	0.170 d
LSD	0.0820

*Mean value in the column sharing similar letters does not differ significantly as determined by the LSD test (P < 0.05).

Table 14. Impact of interaction between treatments and concentrations (T×C) on TSP

Treatments	Total soluble phenols (TSP) µg/g Concentrations (%)		
	0.5%	0.75%	1%
KH_2PO_4	0.3267f	0.3733ef	0.4233def
K_2HPO_4	0.4833cde	0.5300bcd	0.5833abc
Salicylic acid	0.6347ab	0.6833a	0.7233a
Control (DW)	0.1700g	0.1700g	0.1700g
LSD		0.1419	

*Mean value in the column sharing similar letters does not differ significantly as determined by the LSD test (P < 0.05).

***Effect of plant activators on total soluble phenols under field condition*:** Among all the treatments, salicylic acid (0.6723) less TSP value, followed by K_2HPO_4 (0.5222) and KH_2PO_4 (0.3644) µg/g as compared to the control (Table 15). Treatments and concentration interaction (TxC) indicated that salicylic acid expressed more TSP value (0.6277, 0.6730 and 0.7163) followed by K_2HPO_4 (0.4733, 0.5200 and 0.5733) and KH_2PO_4 (0.3167, 0.3633 and 0.4133) µg/g at 0.5%, 0.75% and 1% concentrations as compared to the control (Table 16).

Table 15. Evaluation of plant defense activator against leaf spot of chilli under field condition check the impact of treatment on total soluble phenols.

Treatments	Total soluble phenol (TSP) µg/g
KH_2PO_4	0.364 c
K_2HPO_4	0.522 b
Salicylic Acid	0.672 a
Control (DW)	0.170 d
LSD	0.0816

*Mean value in the column sharing similar letters does not differ significantly as determined by LSD test ($P < 0.05$).

Table 16. Impact of interaction between treatments and concentrations (T×C) on TSP.

Treatments	Total soluble phenols (TSP) µg/g Concentrations (%)		
	0.5%	0.75%	1%
KH_2PO_4	0.317 g	0.363 fg	0.413 efg
K_2HPO_4	0.473 def	0.520 cde	0.573 bcd
Salicylic acid	0.628 abc	0.673 ab	0.716 a
Control (DW)	0.170 h	0.170 h	0.170 h
LSD		0.1413	

*Mean value in the column sharing similar letters does not differ significantly as determined by LSD test ($P < 0.05$).

DISCUSSION

Current study was designed to quantify the alterations in biochemicals (SOD, POD, CAT, H_2O_2, Phenols, TSS and TSP) of both treated and untreated chilli leaves. Maximum (SOD, POD, CAT, H_2O_2, Phenols, TSS and TSP) value was expressed by application of salicylic acid followed by K_2HPO_4 and KH_2PO_4. Antioxidant enzymes like SOD and POD are thought to be the basic biochemical compounds by which plants defend themselves from various biotic and abiotic stresses (Foyer and Shigeoka, 2011). Furthermore, these antioxidants regulate the balance of Reactive oxygen species (Ahmad *et al*., 2008). It is well known that different ROS species including O^{-2} and H_2O_2, are formed during normal cellular reactions and antioxidants repel these ROS species to maintain cell functions (Gill and Tuteja, 2010). The increase in POD is an evidence that treated plants are experiencing an excess of H_2O_2 that is causing H_2O_2 burst because POD is needed to prevent excessive H_2O_2 content and to minimize cellular damage in plants (Liu *et al*., 2014). This circumstance may establish signals of the plants' overall defense mechanisms by alerting biochemical in the plants against potential stress conditions (Zhou *et al*., 2017). CAT activity of the ASM-treated tomato increased as compared to water-sprayed control, showing an overall increased response between 1-72 hrs. Bacterial leaf spot severity was reduced in plants treated by VLA (31.4%) and ASM (49.3%).

Salicylic acid (SA) helps to reduce the degree of clubroot disease by boosting antioxidant enzyme activities (SOD, POD, CAT), osmotic regulation abilities and ROS scavenging capabilities to minimize clubroot-induced damage in pakchoi. Plant activators enhanced the SOD and CAT activities in a delayed enzymatic response typical in compatible host-pathogen interactions. The current study is in line with (Cavalcanti et al., 2006). Previous research found that the expression levels of the genes that code for SOD, POD, CAT and GR have elevated 0.6 mM. (Xi *et al*., 2021).

The SOD activity was dramatically enhanced by the foliar application of acetyl salicylic acid (Hayat *et al*., 2018). Salicylic acid (SA) controls various physiological processes in plants by acting as a possible non-enzymatic antioxidant and plant growth regulator. Plants can activate a defense response and raise the levels of secondary metabolites in response to biotic and abiotic issues such as pathogen attack, physical injury and UV radiation exposure (Ali *et al*., 2006). Increased activity of the enzymes like peroxidase (POD) and phenylalanine ammonia-lyase (PAL) has been observed in plants under various stresses (Neelam *et al*., 2014). According to some theories, SA may impact plant growth under stress by influencing nutrient intake, water relations, stomatal control, and photosynthesis (Hayat *et al*., 2009). It controls the activity of several enzymes, including phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), superoxide dismutase (SOD) and peroxidase (POD), which are key players in triggered plant defense against biotic and abiotic stressors (Zhao *et al*., 2009). Reactive oxygen species (ROS) are produced during an oxidative burst, which is the rapid reaction of plants to elicitor treatments like SA (Kawano, 2003). ROS mediates different signaling pathways to control the expression of genes linked to plant defense mechanisms (Maffei *et al*., 2007). Numerous physiological responses to exogenous applied SA are well recognized, although significant research on SA's impact on phenolic and antioxidative metabolism is still poorly understood.

***Conclusion*:** Salicylic acid (SA) expressed minimum incidence of leaf spot of chilli and expressed alterations in biochemical compounds which are base of creating resistance in chilli plants towards leaf spot disease.

***Authors' Contributions statement*:** Zaid Bin Tahir, Conduct Research Trials, Muhammad Atiq, Conceived the idea and

supervised research work, Nasir Ahmed Rajput, Finalize the manuscript, Azeem Akram : Wrote the original draft of the manuscript. Asif Mehmood Arif, reviewed and edited the manuscript, Shahid Iqbal, Helped in research trials. Shafqat Ali, Helped in research trials. Ahmad Nawaz, Helped with the write-up. Muhammad Usman, Statistical analysis Abuzar Hasnain Helped for table making.

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